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Original article

Piperazine N-substituted naphthyridines, pyridothienopyrimidines and pyridothienotriazines: new antiprotozoals active against *Philasterides dicentrarchi*

José M. Quintela [a], Carlos Peinador [a], Liliana González [a], Raúl Iglesias [b], Anabel Paramá [b], Francisca Álvarez [b], Manuel L. Sanmartín [b], Ricardo Riguera [c,*]

[a] *Departamento de Química Fundamental e Industrial, Facultad de Ciencias, Universidad de La Coruña, La Coruña 15071, Spain*
[b] *Laboratorio de Parasitologia, Instituto de Investigación y Análisis alimentarios, Universidad de Santiago, Santiago de Compostela 15706, Spain*
[c] *Departamento de Química Orgánica, Facultad de Quimica, Instituto de Acuicultura, Universidad de Santiago, Santiago de Compostela 15706, Spain*

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Abstract

New antiprotozoals active against *Philasterides dicentrarchi*, the causative agent of scuticociliatosis in farmed turbot and Black Sea bass-bream, have been synthesised and tested. The most active compounds posses a piperazine ring, generally N-bonded to the heterocycle, and are the 1,8-naphthyridines, **2f** and **5o**, the pyridothienopyrimidine (**7**), and the pyridothienotriazines, **8**, **9**, **12d**, **12f**, **12h**, **12m** and **12k**. Pyridothienotriazine (**12k**) presents the same activity (Lethal Dose, LD = 0.8/1.5 mg L^{-1}) as the well-known antiparasitics niclosamide and oxyclozanide.

Keywords: 1,8-Naphthyridine; Pyridothienopyrimidine; Pyridothienotriazine; Antiprotozoal

1. Introduction

The study of the diseases and disorders found in cultivated fish, crustaceans and molluscs is particularly important due to the ease with which pathogenic agents are transmitted in closed water tanks and the great economic losses incurred from the morbidity and mortality of cultivated marine animals.

In general, the incidence of different parasitic diseases in farm animals is so high that the antiparasitic drug sector is the most important in the animal health market and a very large proportion of the commercial demand for these compounds is related to livestock.

In the field of aquaculture the name scuticociliatosis is well known and is used to identify those diseases of fish, crustaceans and molluscs that are caused by histophagous ciliates of the order Scuticociliatida. The most important pathogenic species belonging to this order include *Uronema marinum*, *Uronema nigricans*, *Anophryoides haemophila* and *Mesanophrys* spp. [1–4]. In the last few years there have been reports of several fatal outbreaks of systemic scuticociliatosis due to *Philasterides dicentrarchi* in farmed sea bass-bream and turbot [5,6]. In the latter case, the incidence of this disease appears to have increased to the extent that it constitutes one of the major parasitic infections in turbot farms and is the cause of significant commercial losses.

For these reasons, screening programs and other efforts to find drugs that are effective against this parasite are especially important. Recently, the in vitro screening of a large number of known pharmaceuticals, selected on the basis of their therapeutic activity in related processes, has been reported [7]. However, really effective control measures remain unknown.

In the course of a study aimed at the discovery of new compounds to combat scuticociliatosis, we centred our efforts on the identification of structural characteristics that could be associated with the desired activity and that could eventually lead to the discovery, design and preparation of effective antiprotozoals.

* Corresponding author.
E-mail address: ricardo@usc.es (R. Riguera).

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In this paper we will show that 1,8-naphthyridines, pyridothienotriazines and pyridotrienodiazines N-bonded to a piperazine residue, are antiprotozoal agents active in vitro against *P. dicentrarchi*.

2. Chemistry

N-heteroaromatic carbaldehydes are extensively used as versatile synthetic building blocks for the preparation of condensed heterocycles [8]. On the basis of this information, we devised synthetic procedures to convert *ortho*-aminoaldehyde (**1**) into 1,8-naphthyridines, **2** and **5**, which are appropriately substituted at position 7. Condensation of the heterocyclic *ortho*-aminoaldehyde (**1**) [9] with aromatic methyl ketones under catalytic alkaline conditions (ethanolic potassium hydroxide) yielded the expected Friedländer products **2a**–**n** as shown in Fig. 1.

On the other hand, condensation of aminoaldehyde (**1**) with ethyl cyanoacetate [10] produced 2-ethoxy-3,6-dicyano-7-hydroxy-4-phenyl-1,8-naphthyridine (**3**), which is easily converted to the chloroderivative **4** by reaction with phosphorus pentachloride.

The 7-chloro-3,6-dicyano-1,8-naphthyridine (**4**) was used as a starting material for the preparation of a series of 7-substituted 1,8-naphthyridines (**5a**–**o**) by nucleophilic halide displacement with nucleophiles (Fig. 2).

Pyrido[2,3-*d*]pyrimidine (**6**) is a new compound and was obtained according to published procedures [11].

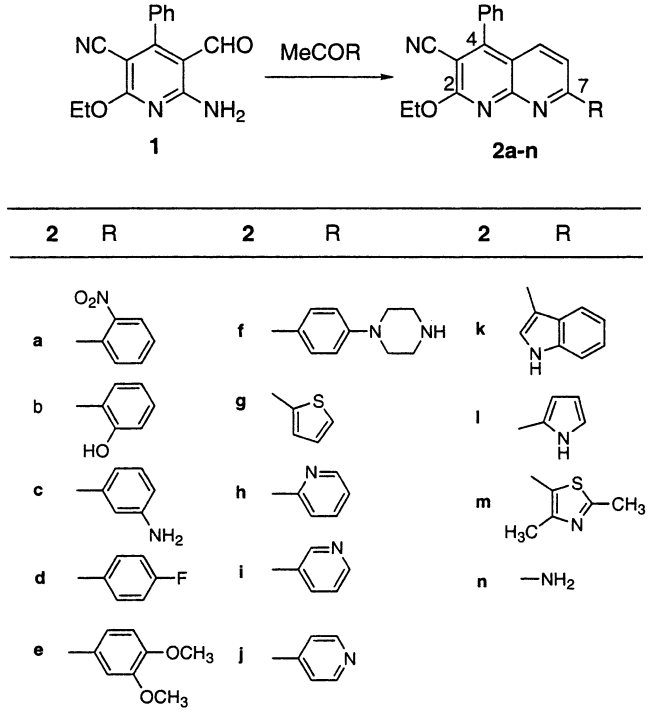


Fig. 1. Synthetic scheme and structures of the 3-cyano-2-ethoxy-4-phenyl-7-substituted-1,8-naphthyridines **2a**–**n**.

Pyridothienopyrimidine (**7**) [12] and pyridothienotriazines, **8** and **9** [13] are previously known compounds and were obtained following methods described in the literature. In the preparation of pyridothienotriazines, **12a**–**p**, which are disubstituted at positions 4 and 7, we decided to introduce the first substituent in the starting material **10** and use the corresponding 4-chloro-derivative **11** as the key intermediate for the introduction of the second substituent by halide displacement with nucleophiles.

Intermediate **11** was prepared by diazotisation of 3-amino-2,5-dicyano-6-substituted thieno[2,3-*b*]pyridines (**10**) [14] and spontaneous intramolecular condensation of the diazonium ion with the adjacent nucleophilic function (Fig. 3).

All the compounds gave satisfactory elemental analyses and spectral data (IR, MS, and ^{1}H- and ^{13}C-NMR) that are consistent with the structures proposed. Details of the synthesis and corresponding experimental data for these compounds are included in the experimental part.

3. Pharmacology

In order to ensure the reliability of the test results for the antiprotozoal activity of our compounds against *P. dicentrarchi*, it was of fundamental importance that the cultivated ciliates remained infective throughout the experiments. To this end, ciliates were harvested by collecting ascitic fluid from the body cavity of naturally infected turbot. The ciliates were then maintained under the culture conditions described by Bernard and Fenchel [15] but with autoclaved *Vibrio anguillarum* as food. Under these conditions, *P. dicentrarchi* retains its capacity to induce scuticociliatosis in experimentally infected turbot.

In addition, the tests were performed in two different media: the standard DMSO–PBS conditions and also in DMSO–filtered sea water. The results obtained in sea water are particularly important because they automatically exclude the possibility of inactivation of the test substance under marine conditions. In fact, a compound found to be effective in vitro by this procedure can also be expected to be effective in bath administration to infected fish or other organisms.

Drugs against *Philasterides* must be able to completely eliminate the ciliates because, due to their extremely high reproduction rate in the fish host, the survival of as few as one to two ciliates per host is enough to cause a second bout of infection. In this particular case, LD99 is a better index of effectivity than LD50 and accordingly, we have expressed our results as LD99: the minimum lethal concentration required to kill 99% of the ciliates.

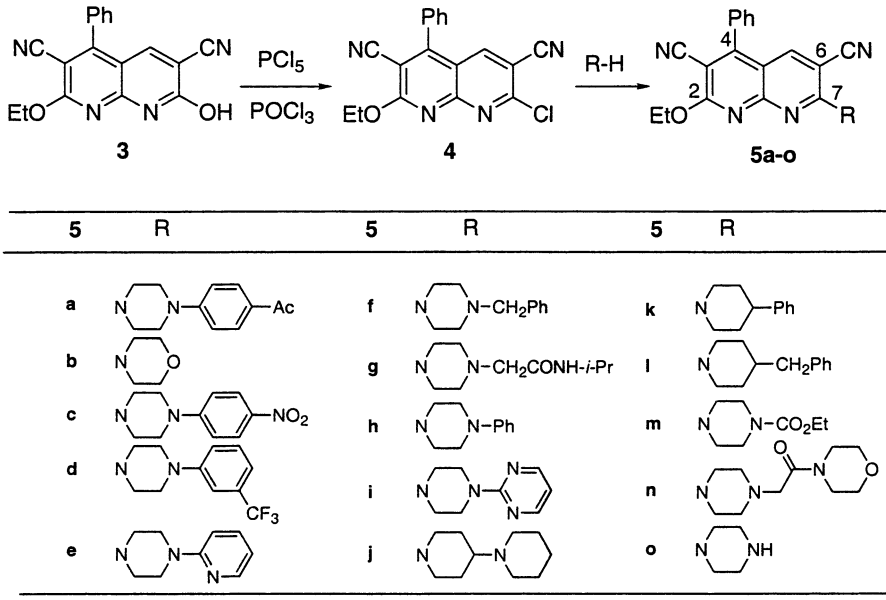
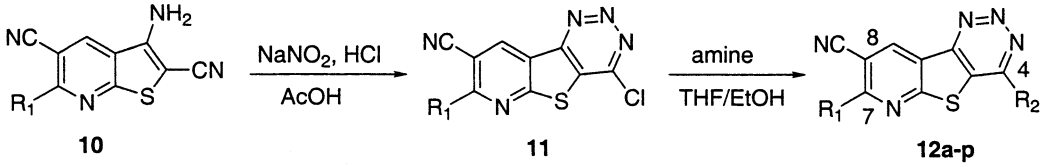


Fig. 2. Synthetic scheme and structures of the 3,6-dicyano-2-ethoxy-4-phenyl-7-substituted-1,8-naphthyridines **5a**–**o**.

4. Results and discussion

When the 1,8-naphthyridines, **2a**–**n**, were submitted to in vitro tests for antiparasitic activity towards the ciliate *P. dicentrarchi* in PBS and in sea water (Table 1), we found that one of the compounds (**2f**) was active in both solvent systems, showing Lethal Dose (LD) values of 50 and 12.5 mg L^{-1}, respectively. Similar testing on 1,8-naphthyridines, **5a**–**o**, revealed that only compound **5o** was active at a similar dose, clearly indicating that the activity is associated with the presence on the 1,8-naphthyridine system of a piperazine substituent and that this unit should preferably have a free NH group.

We attempted to estimate the relative contributions of the different parts of 1,8-naphthyridine and piperazine molecules in the global activity of these compounds. To this end, we tested some simple *N*-phenylpiperazines and found that only those carrying electron-withdrawing substituents (i.e. 2-Cl; 3-Cl; 4-Cl and 3-CF_3) show any activity and, in these cases, it is only moderate (LD = 100 mg L^{-1}). Even this low level of activity is not observed in the unsubstituted *N*-phenylpiperazine either



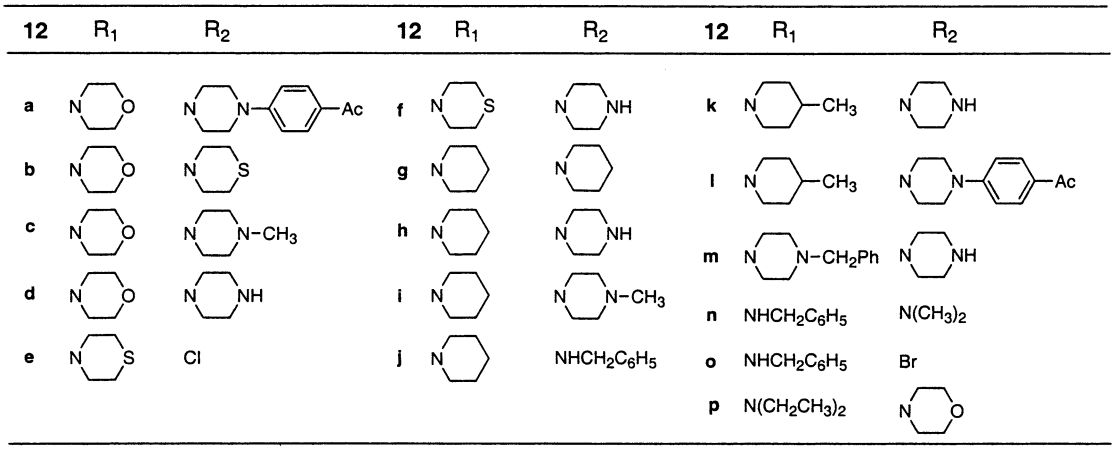


Fig. 3. Synthetic scheme and structures of the 8-cyano-4,7-disubstituted-pyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazines **12a**–**p**.

Table 1
In vitro activity of selected compounds against the protozoal *P. dicenterarchi*

Entry	Compound	Solvent A	Solvent B
1	**2f**	50.0	12.5
2	**5o**	25.0	12.5
3	**7**	12.5	12.5
4	**8**	6.1	3.1
5	**9**	25.0	25.0
6	**12d**	50.0	25.0
7	**12f**	25.0	12.5
8	**12m**	12.5	50.0
9	**12h**	25.0	25.0
10	**12k**	0.8	1.5

The LD (in mg L^{-1}) are measured in DMSO–PBS (solvent A) and in sea water (solvent B).

when the phenyl ring is separated from the piperazine by one, two or three carbons or when the piperazine system is linked to the nitrogen through an acyl group.

All this suggests that the presence of the electron-poor heteroaromatic ring in compounds **2** and **5** is essential for strong antiprotozoal activity. This is consistent with the activity of the well-known antibiotic norfloxazine (LD = 50 mg L^{-1} in both solvents) (Fig. 4) while its analogue lomefloxazine is completely inactive, suggesting that free access to the NH group plays a crucial role in the activity of these compounds.

Replacing the naphthyridine ring of **2f** and **5o**, with other electron-poor nitrogen heterocycles such as the pyridopyrimidine (**6**) (Fig. 4) produced moderately active compound (LD = 100 mg L^{-1}), only but better results were obtained in the pyridothienopyrimidine and the pyridothienotriazine systems. In fact a good level of activity was found in those compounds bearing the piperazine ring, i.e. pyridothienodiazine (**7**) and pyridothienotriazines, **8** and **9**.

Comparison of compounds **8** and **9** indicate that substitution of the ethoxy group at position 7 by a phenyl and of the cyano group at position 8 by hydrogen, produced a 4-fold increase of the LD values.

Replacement in the pyridothienotriazine (**8**) of the phenyl group at position 9 by hydrogen and of the ethoxy group at position 7 by nitrogen nucleophiles produced a new series of compounds **12a**–**p**, which all had significant antiprotozoal activity particularly **12k**, the most active compound found in this work. The structures of representative piperazine-substituted antiprotozoals described in this work are presented in Fig. 4 and the LD values for a selection of the most active compounds (i.e. with LD values of 25 mg L^{-1} or less) are given in Table 1.

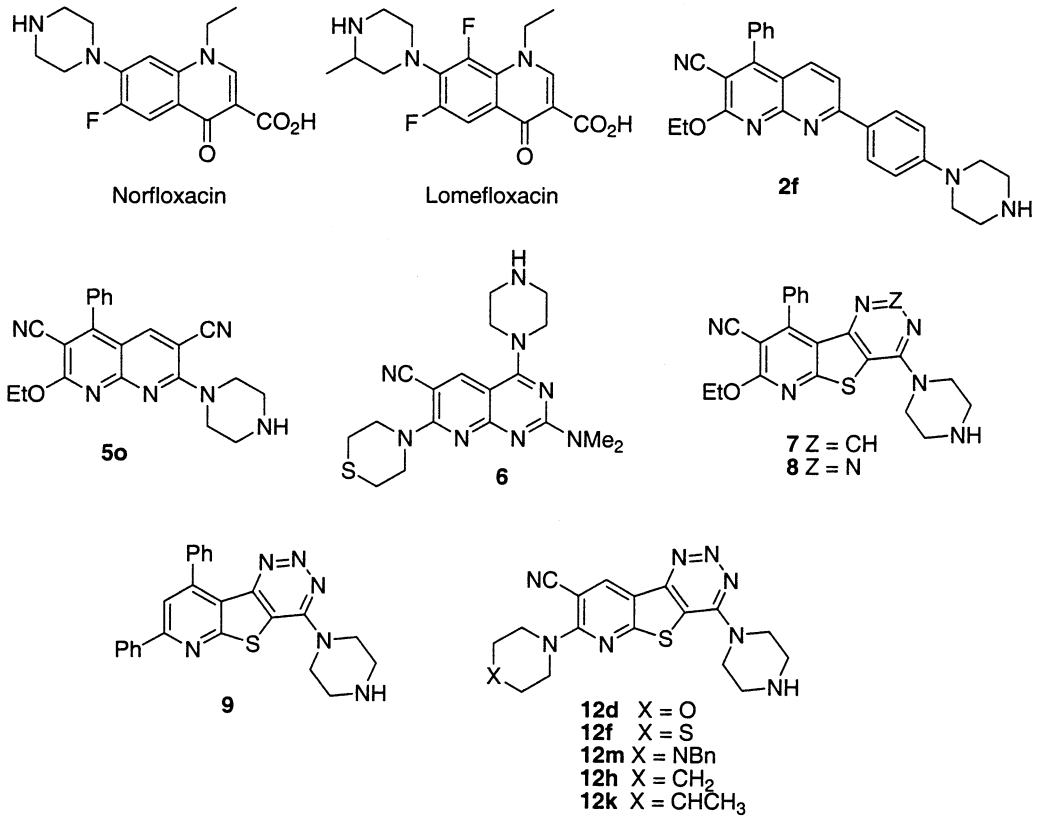


Fig. 4. Structures of representative piperazine-substituted antiprotozoals described in this work.

5. Conclusions

In conclusion, our results demonstrate that 1,8-naphthyridines, pyridothienopyrimidines and pyridothienotriazines incorporating a piperazine group (that keeps a free NH), is directly related to the antiprotozoal activity of the compounds against the ciliate *P. dicentrarchi*. Specific examples of effective new antiparasitic compounds with these structural characteristics include pyridothienopyrimidine (**7**), pyridothienotriazines **8**, **9**, **12d**, **12f**, **12h**, **12m** and, in particular, **12k**—whose LD value (0.8 mg L^{-1} in PBS and 1.5 mg L^{-1} in sea water) is equal to those of other known antiparasitics such as niclosamide and oxyclozanide (0.8 mg L^{-1}) [7]. Studies aimed at improving this activity and the development of other piperazine-substituted heterocycles are currently in progress.

6. Experimental

6.1. Chemistry

All melting points were measured on a Büchi 510 instrument and are uncorrected. IR spectra (potassium bromide) were recorded on a Perkin–Elmer 383 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra (200 and 50 MHz) were recorded on a Bruker AC200F spectrometer. Mass spectra were obtained on a VG4 spectrometer. Microanalyses for C, H and N were performed by the Elemental Analysis General Service of the University of La Coruña. Analyses indicated by symbols of the elements or functions were within $\pm$0.4% of the theoretical values. Silica gel $HF_{254+366}$ for thin layer chromatography and silica gel 60 (230–400 mesh) for flash chromatography were used as purchased from Merck. All reagents were commercial-grade chemicals from freshly opened containers.

6.1.1. 3-Cyano-2-ethoxy-4-phenyl-1,8-napthyridines (**2a**–**m**)

6.1.1.1. General procedure. A solution of **1** (0.75 mmol), and the methyl ketone (or acetonitrile in the case of **2n**) (0.90 mmol) and a catalytic amount of 10% ethanolic potassium hydroxide in ethanol (10 mL) was refluxed until all starting material had disappeared as checked by TLC. After cooling, the precipitate was collected by filtration and recrystallised from a suitable solvent or purified by flash chromatography.

*6.1.1.2. 3-Cyano-2-ethoxy-7-(2-nitrophenyl)-4-phenyl-1,8-naphthyridine (**2a**).* Reaction time: 24 h. Recrystallised from ethanol. Melting point (m.p.) 271–272 °C. Yield: 68%; IR (KBr, cm^{-1}): $\nu = 2240$ (CN); 1585; 1570; 1630; 1330; 1315; 760. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.1$ Hz, CH_3); 4.78 (2H, q, $J = 7.1$ Hz, OCH_2); 7.42 (1H, d, $J = 8.5$ Hz, H-6); 7.74 (8H, m, C_6H_5, C_6H_4); 8.05 (1H, d, $J = 8.5$ Hz, H-5); 8.08 (1H, d, $J = 7.8$ Hz, C_6H_4). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 64.3 (OCH_2); 99.5 (CN); 114.2 (C-3); 116.8; 120.6; 124.6; 129.0; 129.2; 130.0; 130.2; 131.6; 132.8; 133.0; 135.1; 137.0; 148.4; 155.4; 158.4; 161.7; 162.6. MS (EI, *m*/*z*, %): 396 [M^+, 49]. Anal. $C_{23}H_{16}N_4O_3$ (C, H, N).

*6.1.1.3. 3-Cyano-2-ethoxy-7-(2-hydroxyphenyl)-4-phenyl-1,8-naphthyridine (**2b**).* Reaction time: 3 h. Recrystallised from ethanol. M.p.: 259–261 °C. Yield: 69%; IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1590; 1565; 1420; 1330; 1235; 1160; 750. ^{1}H-NMR ($CDCl_3$): $\delta = 1.59$ (3H, t, $J = 7.2$ Hz, CH_3); 4.76 (2H, q, $J = 7.2$ Hz, OCH_2); 6.93–7.13 (2H, m, C_6H_5,); 7.39–7.93 (8H, m, C_6H_5, C_6H_4, H-6); 8.06 (1H, d, $J = 8.8$ Hz, H-5); 14.90 (1H, s, OH). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 64.3 (OCH_2); 114.3 (CN); 115.8; 116.6; 119.0; 119.1; 127.3; 129.1; 129.3; 130.3; 132.8; 133.4; 135.2; 137.3; 153.3; 157.7; 161.7; 162.6; 166.1. MS (FAB, *m*/*z*, %): 368 [(MH^+), 58]. Anal. $C_{23}H_{17}N_3O_2$ (C, H, N).

*6.1.1.4. 7-(3-Aminophenyl)-3-cyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**2c**).* Reaction time: 20 h. Recrystallised from ethanol. M.p.: 276–277 °C. Yield: 67%; IR (KBr, cm^{-1}): $\nu = 3350$ (NH_2); 3000; 2240 (CN); 1590; 1340; 1170; 780. ^{1}H-NMR ($CDCl_3$): $\delta = 1.56$ (3H, t, $J = 7.1$ Hz, CH_3); 3.60 (2H, br s, NH_2); 4.82 (3H, q, $J = 7.1$ Hz, OCH_2); 6.81–7.68 (9H, m, C_6H_5, Py); 7.77 (1H, d, $J = 8.6$ Hz, H-6); 7.99 (1H, d, $J = 8.6$ Hz, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.4$ (CH_3); 64.1 (OCH_2); 98.4 (C-3); 114.6 (CN); 116.6; 118.2; 129.0; 129.3; 129.7; 130.1; 133.2; 136.9; 139.0; 147.0; 156.1; 158.2; 162.7; 162.8. MS (EI, *m*/*z*, %): 366 [M^+, 100]. Anal. $C_{23}H_{18}N_4O$ (C, H, N).

*6.1.1.5. 3-Cyano-2-ethoxy-7-(4-fluorophenyl)-4-phenyl-1,8-naphthyridine (**2d**).* Reaction time: 10 h. Recrystallised from ethanol. M.p.: 205–206 °C. Yield: 79%; IR (KBr, cm^{-1}): $\nu = 2230$ (CN); 1580; 1500; 1380; 1210; 810. ^{1}H-NMR ($CDCl_3$): $\delta = 1.56$ (3H, t, $J = 7.1$ Hz, CH_3); 4.81 (2H, q, $J = 7.1$ Hz, OCH_2); 7.13–7.27 (2H, m, C_6H_4F); 7.46–7.65 (5H, m, C_6H_5); 7.75 (1H, d, $J = 8.5$ Hz, H-6); 8.02 (1H, d, $J = 8.5$ Hz, H-5); 8.17–8.27 (2H, m, C_6H_4F). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 64.2 (OCH_2); 98.6 (CN); 114.4 (C-3); 115.7; 116.1; 116.5; 118.1; 129.0; 129.3; 130.1; 130.2; 133.1; 134.2; 137.2; 155.8; 158.2; 161.5; 162.1; 164.6 (d, $J_{C-F} = 253$ Hz). MS (EI, *m*/*z*, %): 369 [M^+, 55]. Anal. $C_{23}H_{16}N_3OF$ (C, H, N).

*6.1.1.6. 3-Cyano-2-ethoxy-7-(3,4-dimethoxyphenyl)-4-phenyl-1,8-naphthyridine (**2e**).* Reaction time: 1 h. Recrystallised from ethanol. M.p.: 228–229 °C. Yield: 68%; IR (KBr, cm^{-1}): $\nu = 3000$; 2240 (CN); 1590; 1510;

1275; 1160; 1040. ^{1}H-NMR ($CDCl_3$): $\delta = 1.56$ (3H, t, $J = 7.1$ Hz, CH_3); 3.96 (3H, s, OCH_3); 4.06 (3H, s, OCH_3); 4.82 (2H, q, $J = 7.1$ Hz, OCH_2); 7.98 (1H, d, $J = 8.4$ Hz, H-2); 7.46–7.98 (9H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.4$ (CH_3); 56.0 (OCH_3); 56.1 (OCH_3); 64.1 (OCH_2); 98.1 (C-3); 110.9; 114.6 (CN); 116.2; 118.1; 121.2; 129.0; 129.3; 130.1; 130.8; 133.2; 136.8; 149.4; 151.5; 155.9; 158.1; 162.2; 162.8. MS (EI, *m/z*, %): 411 [M^+, 100]. Anal. $C_{25}H_{21}N_3O_3$ (C, H, N).

*6.1.1.7. 3-Cyano-2-ethoxy-4-phenyl-7-(4-piperazinophenyl)-1,8-naphthyridine (**2f**).* Reaction time: 48 h. Recrystallised from ethanol. M.p.: 216–218 °C. Yield: 50%; IR (KBr, cm^{-1}): $\nu = 3240$ (NH); 2220 (CN); 1580; 1320; 1235; 805. ^{1}H-NMR ($CDCl_3$): $\delta = 1.55$ (3H, t, $J = 7.1$ Hz, CH_3); 1.76 (1H, br s NH); 3.06–3.10 (4H, m, NCH_2); 3.31–3.35 (4H, m, NCH_2); 4.81 (2H, q, $J = 7.1$ Hz, OCH_2); 7.01, 7.19 (4H, AA′XX′ system, $J = 9.1$ Hz, C_6H_4); 7.46–7.61 (5H, m, C_6H_5); 7.74 (1H, d, $J = 8.7$ Hz, H-6); 7.92 (1H, d, $J = 8.7$ Hz, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 45.8 (NCH_2); 48.9 (NCH_2); 63.8 (OCH_2); 97.2 (CN); 114.7 (C-3); 116.0; 117.6; 127.9; 128.9; 129.3; 130.0; 133.4; 136.5; 153.3; 156.2; 157.9; 162.2; 162.8. MS (EI, *m/z*, %): 435 [M^+, 45]. Anal. $C_{27}H_{25}N_5O$ (C, H, N).

*6.1.1.8. 3-Cyano-2-ethoxy-4-phenyl-7-(2-thienyl)-1,8-naphthyridine (**2g**).* Reaction time: 20 min. Recrystallised from ethanol. M.p.: 225–226 °C. Yield: 73%; IR (KBr, cm^{-1}): $\nu = 3050$; 2220 (CN); 1595; 1415; 1315; 1205; 800; 700. ^{1}H-NMR ($CDCl_3$): $\delta = 1.55$ (3H, t, $J = 7.1$ Hz, CH_3); 7.81 (2H, q, $J = 7.1$ Hz, OCH_2); 7.15–7.20 (1H, m, thienyl); 7.45–7.69 (7H, m, C_6H_5, thienyl, H-6); 7.83–7.93 (2H, m, thienyl, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.4$ (CH_3); 64.1 (OCH_2); 98.1 (CN); 114.5 (C-3); 116.5; 117.2; 128.1; 128.4; 129.0; 129.3; 130.1; 130.7; 133.1; 136.9; 143.6; 155.9; 157.3; 157.9; 168.9. MS (EI, *m/z*, %): 357 [M^+, 100]. Anal. $C_{21}H_{15}N_3OS$ (C, H, N).

*6.1.1.9. 3-Cyano-2-ethoxy-4-phenyl-7-(2-pyridyl)-1,8-naphthyridine (**2h**).* Reaction time: 20 min. Recrystallised from ethanol. M.p.: 226–228 °C. Yield: 78%; IR (KBr, cm^{-1}): $\nu = 2970$; 2220 (CN); 1575; 1380; 1330; 1160; 1020. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.1$ Hz, CH_3); 4.77 (2H, q, $J = 7.1$ Hz, OCH_2); 7.31–7.60 (6H, m, C_6H_5, Py); 7.78–7.87 (1H, m, Py); 7.99 (1H, d, $J = 8.7$ Hz, H-6); 8.44 (1H, d, $J = 8.7$ Hz, H-5); 8.66–8.71 (2H, m, Py). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 64.0 (OCH_2); 98.9 (C-3); 114.3 (CN); 117.6; 118.5; 122.7; 124.9; 128.9; 129.2; 130.0; 132.9; 136.8; 137.0; 149.1; 154.5; 155.4; 158.2; 161.0; 162.4. MS (EI, *m/z*, %): 352 [M^+, 49]. Anal. $C_{22}H_{16}N_4O$ (C, H, N).

*6.1.1.10. 3-Cyano-2-ethoxy-4-phenyl-7-(3-pyridyl)-1,8-naphthyridine (**2i**).* Reaction time: 3 h. Purified by flash chromatography eluting with dichloromethane–ethanol (99.5/0.5). Yield: 76%; m.p.: 221–222 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1585; 1570; 1445; 1380; 1310; 1215; 1025; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.58$ (3H, t, $J = 7.1$ Hz, CH_3); 4.83 (2H, q, $J = 7.1$ Hz, OCH_2); 7.45–7.54 (3H, m, Py, C_6H_5); 7.60–7.66 (3H, m, C_6H_5); 7.83 (1H, d, $J = 8.8$ Hz, H-6); 8.10 (1H, d, $J = 8.8$ Hz, H-5); 8.56–8.62 (1H, m, Py); 8.73–8.77 (1H, m, Py); 9.35–9.36 (1H, m, Py). ^{13}C-NMR ($CDCl_3$): $\delta = 4.3$ (CH_3); 64.3 (OCH_2); 117.1; 118.2; 123.7; 129.1; 129.3; 130.3; 133.0; 133.8; 135.3; 135.7; 137.7; 149.1; 151.3; 158.4; 160.2. EM (FAB, *m/z*, %): 353 [(MH^+), 100]. Anal. $C_{22}H_{16}N_4O$ (C, H, N).

*6.1.1.11. 3-Cyano-2-ethoxy-4-phenyl-7-(4-pyridyl)-1,8-naphthyridine (**2j**).* Reaction time: 2.5 h. Purified by flash chromatography eluting with dihloromethane–ethanol (99.5/0.5). Yield: 84%; m.p.: 227–228 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1590; 1565; 1385; 1325; 1165; 1020; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.58$ (3H, t, $J = 7.1$ Hz, CH_3); 4.83 (2H, q, $J = 7.1$ Hz, OCH_2); 7.47–7.52 (2H, m, C_6H_5); 7.63–7.66 (3H, m, C_6H_5); 7.84 (1H, d, $J = 8.8$ Hz, H-6); 8.06–8.14 (3H, m, Py, H-5); 8.80–8.83 (2H, m, Py). ^{13}C-NMR δ ($CDCl_3$): $\delta = 14.3$ (CH_3); 64.4 (OCH_2); 114.1 (CN); 117.7; 118.3; 121.9; 129.1; 129.3; 130.4; 132.9; 135.3; 139.9; 145.2; 150.6; 158.4; 158.4; 160.0. MS (FAB, *m/z*, %): 353 [(MH^+), 100]. Anal. $C_{22}H_{16}N_4O$ (C, H, N).

*6.1.1.12. 3-Cyano-2-ethoxy-7-(3-indolyl)-4-phenyl-1,8-naphthyridine (**2k**).* Reaction time: 24 h. Recrystallised from ethanol. M.p.: 261–262 °C. Yield: 45%; IR (KBr, cm^{-1}): $\nu = 3250$ (NH); 2220 (CN); 1600; 1580; 1530; 1210; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.48$ (3H, t, $J = 7.1$ Hz, CH_3); 4.69 (2H, q, $J = 7.1$ Hz, OCH_2); 7.22–7.26 (2H, m, C_6H_4); 7.47–7.70 (8H, m, C_6H_4, C_6H_5, H-6); 7.97 (1H, d, $J = 8.8$ Hz, H-5); 8.43 (1H, s, NH); 8.78–8.82 (1H, m, H-2′). ^{13}C-NMR ($CDCl_3$): $\delta = 14.6$ (CH_3); 63.2 (OCH_2); 95.5 (CN); 112.2; 114.5 (C-3); 114.9; 115.1; 118.5; 121.2; 122.5; 122.6; 125.5; 128.5; 129.0; 129.5; 130.5; 133.2; 135.2; 137.2; 155.8; 157.3; 161.1; 162.1. MS (EI, *m/z*, %): 390 [M^+, 93]. Anal. $C_{25}H_{18}N_4O$ (C, H, N).

*6.1.1.13. 3-Cyano-2-ethoxy-4-phenyl-7-(2-pirrolyl)-1,8-naphthyridine (**2l**).* Reaction time: 30 min. Recrystallised from ethanol. M.p.: 180–182 °C. Yield: 80%; IR (KBr, cm^{-1}): $\nu = 3640$ (NH); 2225 (CN); 1610; 1560; 1330; 1220; 810; 715. ^{1}H-NMR ($CDCl_3$): $\delta = 1.54$ (3H, t, $J = 7.1$ Hz, CH_3); 4.73 (2H, q, $J = 7.1$ Hz, OCH_2); 6.34–6.38 (1H, m, C_4H_3NH); 6.94 (1H, broad singlet, NH); 7.05 (1H, s, C_4H_3NH); 7.43–7.61 (6H, m, C_6H_5, H-6); 7.72 (1H, d, $J = 8.7$ Hz, H-5); 10.3 (1H, s, C_4H_3NH). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 63.9 (OCH_2); 97.0 (CN); 111.2; 112.0; 114.7 (C-3); 115.7; 117.0; 122.9; 128.9; 129.3; 130.0; 130.7; 133.2; 136.3; 154.8; 156.1;

157.8; 163.0. MS (EI, *m/z*, %): 340 [M^+, 100]. Anal. $C_{21}H_{16}N_4O$ (C, H, N).

*6.1.1.14. 7-(2,4-Dimethylthiazol-5-yl)-3-cyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**2m**).* Reaction time: 20 min. Recrystallised from ethanol. M.p.: 235–236 °C. Yield: 71%; IR (KBr, cm^{-1}): $\nu = 2230$ (CN); 1600; 1380; 1330; 810. ^{1}H-NMR ($CDCl_3$): $\delta = 1.55$ (3H, t, $J = 7.1$ Hz, CH_3); 2.73 (3H, s, CH_3); 2.75 (3H, s, CH_3); 4.79 (2H, q, $J = 7.1$ Hz, OCH_2); 7.45–7.62 (6H, m, C_6H_5, H-6); 7.98 (1H, d, $J = 8.6$ Hz, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 17.9 (CH_3); 19.4 (CH_3); 64.2 (OCH_2); 98.5 (CN); 114.4 (C-3); 116.0; 119.3; 129.0; 129.3; 130.2; 131.9; 133.0; 137.0; 152.1; 155.6; 157.0; 158.1; 162.8; 167.3. MS (EI, *m/z*, %): 386 [M^+, 100]. Anal. $C_{22}H_{18}N_4OS$ (C, H, N).

*6.1.1.15. 7-Amino-3-cyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**2n**).* Reaction time: 52 h. Recrystallised from ethanol. M.p.: 268–270 °C. Yield: 53%; IR (KBr, cm^{-1}): $\nu = 3480$, 3100 (NH); 2220 (CN); 1620; 1570; 1420; 1325; 1010. ^{1}H-NMR (DMSO-d_6): $\delta = 1.39$ (3H, t, $J = 7.1$ Hz, CH_3); 4.52 (2H, q, $J = 7.1$ Hz, OCH_2); 6.65 (1H, d, $J = 8.1$ Hz, H-6); 7.30–7.59 (8H, m, C_6H_5, H-5, NH_2); ^{13}C-NMR (DMSO-d_6): $\delta = 14.3$ 9 (CH_3); 62.5 (OCH_2); 90.4; 110.4; 111.6; 115.6 (CN); 128.4; 128.7; 129.1; 129.5; 133.9; 135.8; 156.3; 157.2; 162.1; 162.6. MS (EI, *m/z*, %): 290 [M^+, 100]. Anal. $C_{17}H_{14}N_4O$ (C, H, N).

*6.1.2. 7-Chloro-3,6-dicyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**4**)*

A solution of **3** [3] (0.42 g, 1.3 mmol and phosphorus pentachloride (0.26 g, 1.3 mmol) in phosphorus oxychloride (15 mL) was refluxed for 4 h. The solvent was removed under reduced pressure. The reaction mixture was stirred for 12 h with an ice-cooled solution of water (50 mL) and 10% ammonium hydroxide (15 mL). The solid was filtered off and recrystallised from ethanol. Yield: 0.38 g (85%); m.p.: 238–239 °C. IR (KBr, cm^{-1}): $\nu = 3060$; 2230 (CN); 1580; 1570; 1420, 1355; 1300; 1235; 1150; 1060; 1020; 950. ^{1}H-NMR ($CDCl_3$): $\delta = 1.57$ (3H, t, $J = 7.1$ Hz, CH_3); 4.81 (2H, q, $J = 7.1$ Hz, OCH_2); 7.44–7.70 (5H, m, C_6H_5); 8.30 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 65.7 (OCH_2); 101.4; 107.7 (C-3, C-6); 113.1; 114.2 (CN); 116.0 (CN); 129.1; 129.6; 131.3; 131.4; 144.2; 155.2; 155.9; 158.5; 164.9. MS (EI, *m/z*, %): 334 [M^+, 35]. Anal. $C_{18}H_{11}N_4OCl$ (C, H, N).

*6.1.3. 3,6-Dicyano-2-ethoxy-4-phenyl-1,8-naphthyridines (**5a–o**)*

6.1.3.1. General procedure. A solution of **4** (0.2 g, 0.60 mmol), the appropriate secondary amine (0.63 mmol) and triethylamine (0.16 mL, 1.2 mmol) was refluxed in THF (15 mL) until the starting material had disappeared (TLC). The solvent was removed under reduced pressure and the residue recrystallised from ethanol.

*6.1.3.2. 7-(4-(4-Acetylphenyl)piperazino)-3,6-dicyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**5a**).* Reaction time: 30 min. Yield: 52%, m.p.: 267–269 °C. IR (KBr, cm^{-1}): $\nu = 2220$, 2210 (CN); 1665 (CO); 1600; 1510; 1330; 1230; 805. ^{1}H-NMR ($CDCl_3$): $\delta = 1.52$ (3H, t, $J = 7.1$ Hz, CH_3); 2.53 (3H, s, CH_3); 3.56–3.61 (4H, m, $2CH_2N$); 4.16–4.21 (4H, m, $2CH_2N$); 4.72 (2H, q, $J = 7.1$ Hz, OCH_2); 6.89, 7.91 (m, 4H, C_6H_4); 7.40–7.45 (2H, m, C_6H_5); 7.60–7.63 (3H, m, C_6H_5); 8.06 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 26.1 (CH_3); 46.8 ($2CH_2N$); 47.1 ($2CH_2N$); 64.4 (OCH_2); 94.7 (C-3); 96.1 (C-6); 111.1; 113.3 (CN); 114.2 (CN); 117.2; 128.0; 129.1; 130.4; 130.6; 132.3; 146.8; 153.3; 156.7; 159.1; 165.0; 174.7 (CO). MS (EI, *m/z*, %): 502 [M^+, 5]. Anal. $C_{30}H_{26}N_6O_2$ (C, H, N).

*6.1.3.3. 7-Morpholino-3,6-dicyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**5b**).* Reaction time: 5 h. Yield: 71%; m.p.: 255–256 °C. IR (KBr, cm^{-1}): $\nu = 2860$; 2230, 2210 (CN); 1600; 1510; 1420; 1325; 1120; 1020; 810. ^{1}H-NMR ($CDCl_3$): $\delta = 1.52$ (3H, t, $J = 7.1$ Hz, CH_3); 3.85–3.90 (4H, m, CH_2NCH_2); 3.99–4.04 (4H, m, CH_2OCH_2); 4.72 (2H, q, $J = 7.1$ Hz, OCH_2); 7.39–7.44 (2H, m, C_6H_5); 7.60–7.63 (3H, m, C_6H_5); 8.04 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 48.1 (NCH_2); 64.5 (OCH_2); 66.6 (OCH_2); 94.9 (C-3); 96.1 (C-6); 111.2; 114.3 (CN); 117.3; 129.2; 129.3; 130.6; 132.4; 146.9; 157.8; 159.4; 165.1. MS (EI, *m/z*, %): 385 [M^+, 76]. Anal. $C_{22}H_{19}N_5O_2$ (C, H, N).

*6.1.3.4. 3,6-Dicyano-2-ethoxy-7-[4-(4-nitrophenylpiperazino)]-4-phenyl-1,8-naphthyridine (**5c**).* Reaction time: 24 h. Yield: 93%.; m.p.: 265–266 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1593; 1500; 1445; 1325; 1230; 1110; 1015. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.0$ Hz, CH_3); 3.68 (4H, m, CH_2NCH_2); 4.21 (4H, m, CH_2NCH_2); 4.73 (2H, q, $J = 7.0$ Hz, OCH_2); 6.86, 8.17 (4H, m, C_6H_4); 7.42–7.62 (5H, m, C_6H_5); 8.08 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 46.4 (CH_2NCH_2); 46.9 (CH_2NCH_2); 64.5 (OCH_2); 112.6 (CN); 117.3; 126.0; 129.2; 129.3; 130.7; 132.5; 139.2; 146.9; 154.3. MS (EI, *m/z*, %): 505 [M^+, 9]. Anal. $C_{28}H_{23}N_7O_3$ (C, H, N).

*6.1.3.5. 3,6-Dicyano-2-ethoxy-4-phenyl-7-(4-(3-trifluoromethylphenyl)piperazino]-1,8-naphthyridine (**5d**).* Reaction time: 24 h. Yield: 97%; m.p.: 239–241 °C. IR (KBr, cm^{-1}): $\nu = 2225$ (CN); 1600; 1445; 1400; 1330; 1230; 1110; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.1$ Hz, CH_3); 3.43–3.47 (4H, m, CH_2NCH_2); 4.16–4.20 (4H, m, CH_2NCH_2); 4.74 (2H, q, $J = 7.1$ Hz, OCH_2); 7.10–7.63 (9H, m, C_6H_5, C_6H_4); 8.07 (1H, s, H-

5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 47.4 (NCH_2); 48.5 (NCH_2); 64.4 (OCH_2); 95.0 (C-3); 96.2 (C-6); 111.2; 112.4 (CN); 114.3 (CN); 116.5; 117.3; 119.0; 129.2; 129.8; 130.6; 131.4; 131.9; 132.5; 146.8; 150.9; 156.9; 157.9; 159.4; 165.2. MS (EI, *m/z*, %): 528 [M^+, 5]. Anal. $C_{29}H_{23}N_6OF_3$ (C, H, N).

*6.1.3.6. 3,6-Dicyano-2-ethoxy-4-phenyl-7-[4-(2-pyridylpiperazino)]-1,8-naphthyridine (**5e**).* Reaction time: 20 h. Yield: 100%; m.p.: 300 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1590; 1490; 1430; 1320; 1240; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.84$ (3H, t, $J = 7.1$ Hz, CH_3); 4.07–4.11 (4H, m, CH_2NCH_2); 4.45–4.49 (4H, m, CH_2NCH_2); 5.04 (2H, q, $J = 7.1$ Hz, OCH_2); 6.98–7.03 (2H, m, C_6H_5); 7.72–7.94 (6H, m, C_6H_5, C_5H_4N); 8.36 (1H, s, H-5); 8.53 (1H, d, $J = 6.7$ Hz C_5H_4N). ^{13}C-NMR ($CDCl_3$): $\delta = 14.1$ (CH_3); 44.6 (CH_2NCH_2); 47.1 (CH_2NCH_2); 64.2 (OCH_2); 94.7 (C-3); 95.7 (C-6); 106.8; 110.9; 113.7 (CN); 114.2 (CN); 117.2; 129.1; 130.4; 132.4; 137.5; 146.7; 147.9; 156.7; 157.6; 158.8; 159.2; 165.0. MS (EI, *m/z*, %): 461 [M^+, 3]. Anal. $C_{27}H_{23}N_7O$ (C, H, N).

*6.1.3.7. 3,6-Dicyano-2-ethoxy-7-(4-benzylpiperazino)-4-phenyl-1,8-naphthyridine (**5f**).* Reaction time: 20 h. Yield: 93%; m.p.: 229–230 °C. IR (KBr, cm^{-1}): $\nu =$ 2210 (CN); 1595; 1555; 1500; 1445; 1325; 1250; 1140; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.51$ (3H, t, $J = 7.1$ Hz, CH_3); 2.61–2.65 (4H, m, CH_2NCH_2); 3.59 (2H, s, CH_2); 4.03–4.05 (4H, m, CH_2NCH_2); 4.71 (2H, q, $J = 7.1$ Hz, OCH_2); 7.30–7.61 (10H, m, $2C_6H_5$); 8.00 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.1$ (CH_3); 47.6 (NCH_2); 52.8 (NCH_2); 62.7 ($CH_2C_6H_5$); 64.2 (OCH_2); 94.7 (C-3); 95.6 (C-6); 110.9; 114.4 (CN); 117.4; 127.3; 128.3; 129.2; 130.5; 132.6; 137.5; 146.9; 156.9; 157.7; 159.1; 165.1. MS (EI, *m/z*, %): 474 [M^+, 8]. Anal. $C_{29}H_{26}N_6O$ (C, H, N).

*6.1.3.8. 3,6-Dicyano-2-ethoxy-7-[(4-N-isopropylacetamido)piperazino]-4-phenyl-1,8-napthyridine (**5g**).* Reaction time: 30 min. Yield: 93%; m.p.: 265–266 °C. IR (KBr, cm^{-1}): $\nu = 3280$ (NH); 2220, 2210 (CN); 1645 (CO); 1595; 1325; 1010. ^{1}H-NMR ($CDCl_3$): $\delta = 1.19$ (6H, d, $J = 6.5$ Hz CH_3); 1.52 (3H, t, $J = 7.0$ Hz, CH_3); 2.70–2.71 (4H, m, CH_2NCH_2); 3.06 (2H, s, CH_2CO); 4.01–4.17 (5H, m, CH_2NH_2, CH); 4.71 (2H, q, $J = 7.0$ Hz, OCH_2); 6.84 (1H, br s, NH); 7.39–7.42 (2H, m, C_6H_5); 7.60–7.62 (3H, m, C_6H_5); 8.03 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 22.7 (2 CH_3); 40.7 (NCH_2); 47.7 (NCH_2); 53.1 (CH_2); 61.4 (CH); 64.4 (OCH_2); 95.1 (CN); 97.2 (CN); 111.2; 114.3 (CN); 117.3; 129.2; 129.3; 130.6; 132.5; 146.8; 157.8; 159.5; 165.1; 168.4 (CO). MS (EI, *m/z*, %): 483 [M^+, 6]. Anal. $C_{27}H_{29}N_7O_2$ (C, H, N).

*6.1.3.9. 3,6-Dicyano-2-ethoxy-4-phenyl-7-(4-phenylpiperazino)-1,8-naphthyridine (**5h**).* Reaction time: 20 h. Yield: 85%; m.p.: 276–278 °C. IR (KBr, cm^{-1}): $\nu = 2220$, 2210 (CN); 1595; 1585; 1515; 1325; 1220; 1135; 1020; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.1$ Hz, CH_3); 3.39 (4H, br s, CH_2NCH_2); 4.18 (4H, br s, CH_2NCH_2); 4.73 (2H, q, $J = 7.1$ Hz, OCH_2); 6.89–6.99 (3H, m, C_6H_5); 7.27–7.63 (7H, m, $2C_6H_5$); 8.05 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.1$ (CH_3); 47.5 (NCH_2); 48.9 (NCH_2); 64.3 (OCH_2); 94.8 (C-3); 95.9 (C-6); 110.0; 114.2 (CN); 116.2; 117.3; 120.3; 129.1; 129.2; 130.5; 132.5; 146.8; 150.6; 156.8; 157.7; 159.2; 165.0. MS (EI, *m/z*, %): 460 [M^+, 11]. Anal. $C_{28}H_{24}N_6O$ (C, H, N).

*6.1.3.10. 3,6-Dicyano-2-ethoxy-4-phenyl-7-[4-(2-pyrimidinyl)piperazino]-1,8-naphthyridine (**5i**).* Reaction time: 22 h. Yield: 91%; m.p.: > 300 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1585; 1490; 1440; 1320; 1245; 1135; 975; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J =$ 7.1 Hz, CH_3); 4.04–4.12 (8H, m, CH_2NCH_2); 4.73 (2H, q, $J = 7.1$ Hz, OCH_2); 6.58 (1H, t, $J = 4.8$ Hz, C_6H_3); 7.40–7.64 (5H, m, C_6H_5); 8.06 (1H, s, H-5); 8.37 (2H, d, $J = 4.8$ Hz, C_6H_3). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 43.3 (CH_2NCH_2); 47.5 (CH_2NCH_2); 64.4 (OCH_2); 94.9 (C-3); 95.9 (C-6); 110.5; 111.1; 114.3 (CN); 117.3; 129.2; 130.6; 132.5; 146.8; 156.8; 157.7; 159.4; 161.4; 165.0. MS (EI, *m/z*, %): 462 [M^+, 8]. Anal. $C_{26}H_{22}N_8O$ (C, H, N).

*6.1.3.11. 3,6-Dicyano-2-ethoxy-4-phenyl-7-(4-piperidylpiperidino)-1,8-naphthyridine (**5j**).* Reaction time: 16 h. Yield: 95%; m.p.: 215–217 °C. IR (KBr, cm^{-1}): $\nu = 2930$; 2220, 2210 (CN); 1595; 1515; 1325; 1015; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.44$–1.77 (11H, m, piper); 2.00–2.17 (3H, m, piper); 2.53–2.62 (4H, m, piper); 3.13 (2H, dt, $J = 11.5$ Hz, piper); 4.70 (2H, q, $J =$ 7.1 Hz, OCH_2); 4.80–4.85 (2H, m, piper); 7.38–7.42 (2H, m, C_6H_5); 7.57–7.60 (3H, m, C_6H_5); 7.98 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 24.6, 26.1, 28.0, 40.8, 47.7, 50.1, 62.2 (piper); 64.3 (OCH_2); 94.9 (C-3); 95.4 (C-6); 110.8; 114.5 (CN); 117.4; 129.2; 130.5; 132.6; 146.8; 157.0; 157.6; 159.1; 165.0. MS (EI, *m/z*, %): 466 [M^+, 3]. Anal. $C_{28}H_{30}N_6O$ (C, H, N).

*6.1.3.12. 3,6-Dicyano-2-ethoxy-4-phenyl-7-(4-phenylpiperidino)-1,8-naphthyridine (**5k**).* Reaction time: 16 h. Yield: 95%; m.p.: 273–275 °C. IR (KBr, cm^{-1}): $\nu = 2910$; 2220 (CN); 1595; 1470; 1450; 1325; 1240; 1005; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.45$ (3H, t, $J =$ 7.1 Hz, CH_3); 1.79–2.03 (4H, m, piper); 2.79–2.95 (1H, m, piper); 3.19 (2H, dd, $J = 11.9$ Hz, piper); 4.65 (2H, q, J = 7.1 Hz, OCH_2); 4.88 (2H, dd, $J = 13.5$ Hz, piper); 7.16–7.56 (10H, m, $2C_6H_5$); 7.95 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 33.3, 42.6, 48.8 (piper); 64.3 (OCH_2); 95.0 (C-3); 95.5 (C-6); 110.8; 114.5 (CN); 117.5; 126.6; 126.8; 128.6; 129.2; 130.5; 132.6; 144.9; 146.9; 157.0; 157.7; 159.4; 165.1. MS (EI, *m/z*, %): 459 [M^+, 39]. Anal. $C_{29}H_{25}N_5O$ (C, H, N).

*6.1.3.13. 7-(4-Benzylpiperidino)-3,6-dicyano-2-ethoxy-4-phenyl-1,8-naphthyridine (**5l**).* Reaction time: 16 h. Yield: 91%; m.p.: 204–205 °C. IR (KBr, cm^{-1}): $\nu =$ 2910; 2220, 2210 (CN); 1600; 1450; 1325; 1130; 1015; 795. ^{1}H-NMR ($CDCl_3$): $\delta = 1.41–1.63$ (2H, m, piper); 1.51 (3H, t, $J = 7.1$ Hz, CH_3); 1.84–1.88 (3H, m, piper); 2.61 (2H, d, $J = 6.6$ Hz, CH_2); 3.09 (2H, t, $J = 12.6$ Hz, piper); 4.66–4.79 (2H, m, piper); 4.70 (2H, q, $J = 7.1$ Hz, OCH_2); 7.15–7.61 (10H, m, $2C_6H_5$); 7.98 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 32.1 (CH_2); 38.2, 42.9, 48.4 (piper); 64.2 (OCH_2); 94.9 (C-3); 95.3 (C-6); 110.7; 114.5 (CN); 117.5; 126.1; 128.3; 129.0; 129.1; 130.5; 132.6; 139.8; 146.8; 157.0; 157.6; 159.2; 165.2. MS (EI, *m/z*, %): 473 [M^+, 39]. Anal. $C_{30}H_{27}N_5O$ (C, H, N).

*6.1.3.14. 3,6-Dicyano-2-ethoxy-7-(4-ethoxycarbonylpiperazino)-4-phenyl-1,8-naphthyridine (**5m**).* Reaction time: 24 h. Yield: 65%; m.p.: 273–275 °C. IR (KBr, cm^{-1}): $\nu = 2990$; 2220, 2210 (CN); 1700 (CO); 1595; 1430; 1245; 1010; 800. ^{1}H-NMR ($CDCl_3$): $\delta = 1.29$ (3H, t, $J = 7.1$ Hz, CH_3); 1.52 (3H, t, $J = 7.1$ Hz, CH_3); 3.67–3.72 (4H, m, NCH_2); 3.96–4.00 (4H, m, NCH_2); 4.20 (2H, q, $J = 7.1$ Hz, OCH_2); 4.72 (2H, q, $J = 7.1$ Hz, OCH_2); 7.39–7.44 (2H, m, C_6H_5); 7.59–7.63 (3H, m, C_6H_5); 8.05 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.3$ (CH_3); 14.6 (CH_3); 43.3 (NCH_2); 47.6 (NCH_2); 61.8 (OCH_2); 64.5 (OCH_2); 95.1 (C-3); 96.2 (C-6); 112.2; 114.3 (CN); 117.2; 129.1; 129.2; 129.6; 130.6; 132.4; 146.8; 155.4; 156.8; 157.8; 159.6; 165.1. MS (EI, *m/z*, %): 456 [M^+, 21]. Anal. $C_{25}H_{24}N_6O_3$ (C, H, N).

*6.1.3.15. 3,6-Dicyano-2-ethoxy-7-(4-(2-oxo-2-morpholinoethyl)piperazino)-4-phenyl-1,8-naphthyridine (**5n**).* Reaction time: 3 h. Yield: 93%; m.p.: 211–213 °C. IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1635 (CO); 1595; 1500; 1440; 1325; 1110; 1000. ^{1}H-NMR ($CDCl_3$): $\delta = 1.51$ (3H, t, $J = 7.1$, Hz, CH_3); 2.71–2.75 (4H, m, CH_2NCH_2); 3.27 (2H, s, CH_2CO); 3.63–3.67 (8H, m, CH_2NCH_2, CH_2OCH_2); 4.01–4.03 (4H, m, CH_2NCH_2); 4.69 (2H, q, $J = 7.1$ Hz, OCH_2); 7.38–7.61 (5H, m, C_6H_5); 8.01 (1H, s, H-5). ^{13}C-NMR ($CDCl_3$): $\delta = 14.2$ (CH_3); 42.1 (CH_2CO); 46.2 (CH_2NCH_2); 47.6 (CH_2NCH_2); 52.8; 60.4; 64.4 (OCH_2); 66.8 (CH_2OCH_2); 66.9 (CH_2OCH_2); 94.9 (C-3); 95.9 (C-6); 111.0; 114.3 (CN); 117.3; 129.1; 129.2; 130.5; 132.4; 146.8; 156.8; 157.7; 159.2; 165.0; 167.5 (CO). MS (EI, *m/z*, %): 511 [M^+, 3]. Anal. $C_{28}H_{29}N_7O_3$ (C, H, N).

*6.1.3.16. 3,6-Dicyano-2-ethoxy-4-phenyl-7-piperazino-1,8-naphthyridine (**5o**).* Reaction time: 30 min. Yield: 70%; m.p.: 182–184 °C. IR (KBr, cm^{-1}): $\nu = 3434$ (NH); 2224 (CN); 1586; 1571; 1542; 1513; 1445; 1423; 1404; 1384; 1329; 1255; 1231; 1133; 1113; 1018. ^{1}H-NMR ($CDCl_3$): $\delta = 1.53$ (3H, t, $J = 7.0$ Hz, CH_3); 3.07 (4H, t, $J = 5.0$ Hz, NCH_2); 3.99 (4H, t, $J = 5.0$ Hz, NCH_2); 4.72 (2H, q, $J = 7.0$ Hz, OCH_2); 7.39–7.44 (2H, m, C_6H_5); 7.55–7.79 (3H, m, C_6H_5); 8.02 (1H, m, H-5). ^{13}C-NMR (DMSO-d_6): $\delta = 14.3$ (CH_3); 46.0 (NCH_2); 49.0 (NCH_2); 64.4 (OCH_2); 94.9 (C-3); 95.9 (C-6); 110.9, 114.4 (CN); 117.4; 129.2, 130.6, 132.5 (C_6H_5); 146.9 (C-5); 157.7; 159.4; 165.1. MS (EI, *m/z*, %): 384 [M^+, 2]. Anal. $C_{22}H_{20}N_6O$ (C, H, N).

*6.1.4. 6-Cyano-2-dimethylamino-4-piperazino-7-thiomorpholinopyrido[2,3-d]pyrimidine (**6**)*

Obtained by a previously reported procedure [11]. Yield: 60%; m.p.: 300 °C. IR (KBr, cm^{-1}). $\nu = 2200$ (CN); 1600; 1575; 1550; 1500; 1440; 1425. ^{1}H-NMR ($CDCl_3$). $\delta = 2.77$ (4H, m, CH_2S); 3.18 (s, 6H, NMe_2); 3.23 (m, 4H, NCH_2); 3.85 (m, 8H, NCH_2); 8.47 (s, 1H, H-5); 9.50 (broad singlet exchangeable with D_2O, 1H, NH). ^{13}C-NMR (DMSO-d_6): $\delta = 26.5$ (CH_2S); 37.1 (NMe_2); 42.3; 45.6; 49.9 (NCH_2); 87.4 (C-6); 97.9 (C-4a); 118.1 (CN); 144.9 (C-5); 160.1; 163.1. MS (EI, *m/z*, %): 384 [M^+, 19]. Anal. $C_{18}H_{24}N_8S$ (C, H, N).

*6.1.5. Synthesis of Pyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazines (**12a–p**)*

6.1.5.1. General procedure. To an ice-cooled solution of the corresponding β-enaminonitrile (**10**) [14] (6.73 mmol) in 1:1 (v/v) HCl–AcOH or HBr–AcOH (50 mL) a solution of sodium nitrite (10.1 mmol) in water (10 mL) was added dropwise. The solution was stirred at room temperature for 3 h, and the mixture was poured into water (200 mL). The resulting solid was filtered off and purified by flash chromatography on silica gel to yield **11**. A solution of the corresponding halogenated derivative **11** (0.60 mmol) and the appropriate secondary amine (0.72 mmol) in 3:1(v/v) THF–EtOH (15 mL) was refluxed until the starting material disappeared (TLC). After cooling, the solid was filtered off and recrystallised or purified by flash chromatography. Compounds **12a**, **12b**, **12d**, **12l** and **12o** have already been described [14].

*6.1.5.2. 8-Cyano-4-(4-methylpiperazino)-7-morpholinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12c**).* Reaction time: 3 h. Recrystallised from EtOH–acetone. M.p.: 294–296 °C. Yield: 74%; IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1590; 1540; 1275. ^{1}H-NMR (DMSO-d_6): $\delta = 2.77$ (3H, s, CH_3); 3.79 (16H, m, NCH_2, CH_2O); 9.10 (1H, s, H-9). ^{13}C-NMR (DMSO-d_6): $\delta = 42.3$ (CH_3); 48.2 (NCH_2, CH_2); 51.8 (NCH_2); 65.8 (CH_2O); 93.8 (C-8); 111.7 (C-4a); 115.9 (CN); 117.4 (C-9a); 140.8 (C-9); 149.1; 152.5; 160.0; 163.8. MS (FAB, *m/z*, %): 397 [$(MH)^+$, 100]. Anal. $C_{18}H_{20}N_8OS$ (C, H, N).

*6.1.5.3. 4-Chloro-8-cyano-7-thiomorpholinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12e**).* Reaction time: 0.5 h. Purified by flash chromatography using CH_2Cl_2 as eluent. Yield: 94%. ^{1}H-NMR ($CDCl_3$): $\delta = 2.88$ (4H, t, $J = 5.1$ Hz, SCH_2); 4.31 (4H, t, $J = 5.1$ Hz, NCH_2); 8.93 (1H, s, H-9). ^{13}C-NMR ($CDCl_3$): $\delta = 27.4$ (SCH_2); 51.1 (NCH_2); 94.1 (C-8); 115.3 (C-4a); 116.9 (CN); 129.6 (C-9a); 141.6 (C-9); 150.9; 152.4; 159.8; 165.6. MS (FAB, *m/z*, %): 317 [$(MH)^+$, 100]. Anal. $C_{13}H_9N_6S_2Cl$ (C, H, N).

*6.1.5.4. 8-Cyano-4-piperazino-7-thiomorpholinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12f**).* Reaction time: 1 h. Recrystallised from EtOH. M.p.: 192–194 °C. Yield: 84%; ^{1}H-NMR (DMSO-d_6): $\delta = 2.82$ (4H, m, SCH_2); 4.09–4.18 (8H, m, NCH_2); 9.03 (1H, b.s., H-9); 9.56 (1H, b.s. exchangeable with D_2O, NH). MS (FAB, *m/z*, %): 399 [$(MH)^+$, 100]. Anal. $C_{17}H_{18}N_8S_2$ (C, H, N).

*6.1.5.5. 8-Cyano-4,7-dipiperidinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12g**).* Reaction time: 5 h. Recrystallised from EtOH–acetone. M.p.: 228–230 °C. Yield: 68%; IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1600; 1550–1535; 1500; 1450; 1360; 1310; 1270; 1245. ^{1}H-NMR ($CDCl_3$): $\delta = 1.79$ (12H, m, CH_2); 3.86 (4H, m, NCH_2); 4.03 (4H, m, NCH_2); 8.79 (1H, s, H-9). ^{13}C-NMR ($CDCl_3$): $\delta = 24.3$, 24.4, 25.8, 26.0 (CH_2); 47.3, 49.5 (NCH_2); 93.2 (C-8); 110.9 (C-4a); 116.1 (CN); 117.7 (C-9a); 140.2 (C-9); 148.9; 152.5; 160.0; 164.4. MS (EI, *m/z*, %): 379 [M^+, 19]. Anal. $C_{19}H_{21}N_7S$ (C, H, N).

*6.1.5.6. 8-Cyano-4-piperazino-7-piperidinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12h**).* Reaction time: 1 h. Recrystallised from EtOH. M.p.: 281–283 °C. Yield: 80%; ^{1}H-NMR (DMSO-d_6): $\delta = 1.68$ (6H, m, CH_2); 3.81 (4H, m, NCH_2); 4.17 (4H, m, NCH_2); 8.99 (1H, s, H-9); 9.46 (1H, b.s., NH). ^{13}C-NMR (DMSO-d_6): $\delta = 23.7$, 25.4 (CH_2); 42.2, 42.4, 49.0 (NCH_2); 93.2 (C-8); 111.3 (C-4a); 115.1 (CN); 117.5 (C-9a); 140.6 (C-9); 149.0; 152.4; 159.8; 163.9. MS (FAB, *m/z*, %): 381 [$(MH)^+$, 100]. Anal. $C_{18}H_{20}N_8S$ (C, H, N).

*6.1.5.7. 8-Cyano-4-(4-methylpiperazino)-7-piperidinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12i**).* Reaction time: 3 h. Recrystallised from EtOH–acetone. M.p.: 270 °C (dec.). Yield: 70%; IR (KBr, cm^{-1}): $\nu = 2240$ (CN); 1610; 1560; 1540; 1510; 1460; 1420; 1410; 1370; 1320; 1280; 1260. ^{1}H-NMR (DMSO-d_6): $\delta = 1.68$ (6H, m, CH_2); 2.79 (3H, s, CH_3); 3.79 (4H, m, NCH_2); 4.14–4.29 (8H, m, NCH_2); 8.91 (1H, s, H-9). ^{13}C-NMR (DMSO-d_6): $\delta = 23.7$, 25.4 (CH_2); 42.2 (CH_3); 42.6, 49.0, 51.6 (NCH_2); 93.2 (C-8); 111.3 (C-4a); 115.0 (CN); 117.5 (C-9a); 140.6 (C-9); 149.1; 152.3; 159.8; 163.9. MS (FAB, *m/z*, %): 395 [$(MH)^+$, 100]. Anal. $C_{19}H_{22}N_8S$ (C, H, N).

*6.1.5.8. 4-Benzylamino-8-cyano-7-piperidinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12j**).* Reaction time: 13 h. Recrystallised from EtOH–acetone. Yield: 50%. IR (KBr, cm^{-1}): $\nu = 3635$ (NH); 2220 (CN); 1595; 1540; 1510; 1495; 1430; 1300; 1250. ^{1}H-NMR ($CDCl_3$): $\delta = 1.77$ (6H, m, CH_2); 3.87 (4H, m, NCH_2); 5.02 (2H, d, $J = 5.7$ Hz, $NHCH_2$); 5.41 (1H, t, $J = 5.7$ Hz, NH); 7.32–7.48 (5H, m, C_6H_5); 8.78 (1H, s, H-9). ^{13}C-NMR ($CDCl_3$): $\delta = 24.3$, 25.9 (CH_2); 45.6, 49.5 (NCH_2); 93.3 (C-8); 112.0 (C-4a); 116.5 (CN); 117.7 (C-9a); 128.1, 128.2, 128.9, 137.3 (C_6H_5); 140.5 (C-9); 151.9; 159.9; 164.9. MS (EI, *m/z*, %): 401 [M^+, 4]. Anal. $C_{21}H_{19}N_7S$ (C, H, N).

*6.1.5.9. 8-Cyano-7-(4-methylpiperidino)-4-piperazinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12k**).* Reaction time: 1 h. Recrystallised from EtOH–acetone. M.p.: 213–215 °C. Yield: 97%; IR (KBr, cm^{-1}): $\nu = 3400$ (NH); 2215 (CN); 1597; 1551; 1533; 1495; 1445; 1405; 1364; 1314; 1276; 1258; 1245. ^{1}H-NMR (DMSO-d_6): $\delta = 0.94$ (3H, d, $J = 6.0$ Hz, CH_3); 1.27 (2H, m, CH_2); 1.77 (3H, m, CH_2, CH); 2.90 (4H, m, NCH_2); 3.13 (2H, m, NCH_2); 3.89 (4H, m, NCH_2); 4.42 (2H, m, NCH_2); 8.85 (1H, s, H-9). ^{13}C-NMR (DMSO-d_6): $\delta = 21.5$ (CH_3); 30.1 (CH); 33.6, 42.8 (CH_2); 46.2, 48.3 (NCH_2); 93.1 (C-8); 110.4 (C-4a); 115.5 (CN); 117.5 (C-9a); 140.3 (C-9); 148.5; 152.3; 159.7; 163.8. MS (EI, *m/z*, %): 394 [M^+, 6]. Anal. $C_{19}H_{22}N_8S$ (C, H, N).

*6.1.5.10. 7-(4-Benzylpiperazino)-8-cyano-4-piperazinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12m**).* Reaction time: 0.5 h. Recrystallised from EtOH. M.p.: 215–217 °C (dec.). Yield: 66%; IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1600; 1550; 1530; 1490; 1440; 1360–1340; 1310; 1270; 1250. ^{1}H-NMR (DMSO-d_6): $\delta =$ 2.56 (4H, b.s., NCH_2); 3.23 (4H, m, NCH_2); 3.56 (2H, s, CH_2); 3.82 (4H, m, NCH_2); 4.11 (4H, m, NCH_2); 7.33 (5H, m, C_6H_5); 9.02 (1H, s, H-9). Anal. $C_{24}H_{25}N_9S$ (C, H, N).

*6.1.5.11. 7-Benzylamino-8-cyano-4-dimethylaminopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12n**).* Reaction time: 2 h. Purified by flash chromatography using as eluent CH_2Cl_2–AcOEt 20:1 (v/v). M.p.: 198–200 °C. Yield: 60%; IR (KBr, cm^{-1}): $\nu = 3351$ (NH); 2217 (CN); 1597; 1560; 1518; 1453; 1428; 1382; 1345; 1323; 1297; 1239; 1114; 1084. ^{1}H-NMR (DMSO-d_6): $\delta = 3.34$ (6H, s, NCH_3); 4.63 (2H, d, $J =$ 5.4 Hz, CH_2); 7.21–7.34 (5H, m, C_6H_5); 8.57 (1H, m, NH); 8.74 (1H, s, H-9). ^{13}C-NMR (DMSO-d_6): $\delta = 38.3$ (NCH_3); 44.5 (CH_2); 91.5 (C-8); 109.3 (C-4a); 114.2 (CN); 116.0 (C-9a); 126.8, 127.4, 128.3, 138.9 (C_6H_5);

138.2 (C-9); 148.1; 152.7; 158.1; 165.1. Anal. $C_{18}H_{15}N_7S$ (C, H, N).

*6.1.5.12. 8-Cyano-7-diethylamino-4-morpholinopyrido[3′,2′:4,5]thieno[3,2-d]-1,2,3-triazine (**12p**).* Reaction time: 3 h. Recrystallised from EtOH–acetone. M.p.: 229–231 °C. Yield: 83%; IR (KBr, cm^{-1}): $\nu = 2220$ (CN); 1600; 1560; 1510; 1450; 1430; 1385; 1360; 1335; 1320; 1270; 1250. ^{1}H-NMR ($CDCl_3$): $\delta = 1.36$ (6H, t, $J = 7.1$ Hz, CH_3); 3.82–3.92 (8H, m, NCH_2); 4.04 (4H, m, OCH_2); 8.78 (1H, s, H-9). ^{13}C-NMR ($CDCl_3$): $\delta = 13.4$ (CH_3); 44.9, 46.0 (NCH_2); 66.5 (OCH_2); 90.1 (C-8); 110.9 (C-4a); 114.5, 118.5 (CN, C-9a); 141.0 (C-9); 149.5; 152.8; 157.2; 164.8. MS (EI, *m*/*z*, %): 369 [M^+, 88]. Anal. $C_{17}H_{19}N_7OS$ (C, H, N).

6.2. Pharmacology

6.2.1. Isolation and culture of P. dicentrarchi

Ciliates were harvested by collecting ascitic fluid from the body cavity of naturally infected turbot and were maintained under the culture conditions described by Bernard and Fenchel [15] with autoclaved *V. anguillarum* as food.

6.2.2. Determination of lethal activity

Stock solutions of the synthesised compounds were prepared in distilled water or dimethylsulphoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA). The resulting stocks were diluted in physiological phosphate-buffered saline (PBS; pH 7.2) or filtered (0.2 μm) sea water (salinity 28%) to give the final concentrations used in the screening (see below). Tests performed in filtered sea water allow automatic exclusion of the possibility of partial or total inactivation of the test substance under marine conditions.

Ciliates in the late exponential phase or early plateau phase of culture were concentrated by centrifugation at $650 \times g$ for 5 min and then resuspended in PBS or filtered sea water. After counting in a haemocytometer, 10 μL of ciliate suspension containing 10^4 ciliates was added to each well of a 96-well microtitre polystyrene plate containing 90 μL per well of the candidate antiprotozoal at the required dose (100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0.7 mg L^{-1}) in PBS or filtered sea water. Plates were then incubated at 18 °C for 24 h.

Each determination was performed in duplicate. Wells containing ciliates in assay solution (PBS or filtered sea water) without chemicals were also assayed as negative controls. In order to rule out possible effects of the solvent in samples dissolved in DMSO, duplicate wells with PBS or filtered sea water containing the highest concentration of DMSO used (up to 2.5%) were also included.

Ciliate motility after incubation was checked using an inverted microscope with phase-contrast illumination. Prior to scanning, each culture plate was gently rocked to ensure uniform distribution of ciliates throughout the medium. The results are expressed as LD: the minimum lethal concentration required to kill 99% of the ciliates.

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